Inhibition of the Transforming Growth Factor-β/Smad Signaling Pathway in the Epithelium of Oral Lichen

Andreas Karatsaidis, Olav Schreurs, Tony Axéll,* Kristen Helgeland, and Karl Schenck
Department of Oral Biology and *Department of Clinical Dentistry, Dental Faculty, University of Oslo, Oslo, Norway

The basal cells in epithelium of the erythematous form of oral lichen display hyperproliferation compared with normal oral mucosa. In this study we examined whether this is associated with disrupted production, activation, or signal transduction of the epithelial growth inhibitor transforming growth factor (TGF) β1. *In situ* immunostaining showed that most epithelial cells in normal oral mucosa had nuclear and cytoplasmic Smad4 and phosphorylated Smad2/3, but expressed little or no Smad7. Expression of latency-associated peptide TGF-β1, latent TGF-β binding protein 1, TGF-β type I receptor, and TGF-β type II receptor was readily seen, but only very little TGF-β1 was activated. In erythematous oral lichen, basal and lower spinous epithelial layers showed staining for latency-associated peptide TGF-β1, TGF-β type I receptor, and TGF-β type II receptor. A band with scanty staining for these molecules, but with marked staining for active TGF-β1, was seen in the upper spinous and granular layers. Numbers of epithelial cell nuclei with Smad4 and phosphorylated Smad2/3 staining were significantly reduced in erythematous oral lichen compared with normal oral mucosa. Basal and suprabasal cell layers in erythematous oral lichen showed strong cytoplasmic Smad7 protein staining, but in spinous and granular layers Smad7 was localized to the cell membrane. *In situ* hybridization showed strong Smad7 mRNA expression in almost all basal keratinocytes in erythematous oral lichen; by contrast, no or occasionally very weak Smad7 mRNA expression was seen in these cells in normal oral mucosa. The observations indicate that inhibition of the TGF-β/Smad pathway may account for the hyperproliferation of keratinocytes in erythematous oral lichen. ***Key words: epithelial proliferation/keratinocytes/oral mucosa. J Invest Dermatol 121:00–00, 2003***

Oral lichen (OL), which includes oral lichenoid reactions and oral lichen planus (OLP), is a common oral disease affecting about 2% of the populations examined (Axell and Rundquist, 1987; Salonen *et al*, 1990). Oral lichenoid reactions are defined as adverse reactions to dental materials, whereas changes with unknown etiology are termed OLP (Bolewska *et al*, 1990). Histologically, oral lichenoid reactions and OLP are indistinguishable from each other (Bolewska and Reibel, 1989). The lesions are characterized by a typical band-like mononuclear inflammatory infiltrate in the connective tissue, dominated by T lymphocytes (Hedberg *et al*, 1986). Pathologic changes in the epithelium include hyperorthokeratosis or parakeratosis, acanthosis, atrophy, and liquefaction degeneration of basal cells (Andreasen, 1968; Kramer *et al*, 1978). Clinically, OL shows white papular, reticular, erythematous, plaque, and ulcerative forms (Andreasen, 1968; Kramer *et al*, 1978). Erythematous OL (ERY OL) is one of the common forms of the disease and is often associated with oral discomfort in the affected persons (Andreasen, 1968).

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Reprint requests to: Andreas Karatsaidis, Department of Oral Biology, PO Box 1052, Blindern, N-0316 Oslo, Norway. Email: akaratsa@odont.uio.no

Abbrevations: ERY OL, erythematous oral lichen; LAP-TGF-β1, latency-associated peptide TGF-β1; LTBP-1, latent TGF-β binding protein 1; NOM, normal oral mucosa; OL, oral lichen; OLP, oral lichen planus; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor.

In order to maintain the stratified epithelium's normal anatomy and thereby its functions, there is a fine-tuned balance between the continuous proliferation of basal keratinocytes and maturation and cell death of terminally differentiating keratinocytes (Gandarillas, 2000). Epithelial proliferation and differentiation are regulated by an intricate signaling network of different peptides (cytokines/growth factors) produced by keratinocytes themselves, stromal cells, and infiltrating inflammatory cells and their respective receptors (Dotto, 1999; Freedberg *et al*, 2001). Transforming growth factor β (TGF-β) has been shown to have important regulatory functions in epithelial proliferation and differentiation (Roberts, 1998; Ten Dijke *et al*, 2002). Among the three TGF-β isoforms, TGF-β1 is known as the most prominent regulator. TGF-β1 inhibits epithelial cell growth through transcriptional repression of the growth promoting gene *c-myc* (Pietenpol *et al*, 1990), and upregulates the cyclin-dependent kinase inhibitors p15 and p21 (Hannon and Beach, 1994; Datto *et al*, 1995). This leaves the retinoblastoma protein in a hypo-phosphorylated state and thereby retains the cells in the G1 phase (Weinberg, 1995). TGF-β1 can also induce apoptosis in keratinocytes (Min *et al*, 1999), and affect keratinocyte differentiation by upregulating the expression of integrins and keratins (Mansbridge and Hanawalt, 1988; Jiang *et al*, 1995; Zambruno *et al*, 1995). The crucial role of TGF-β1 for epithelial development is highlighted in studies on transgenic mice: the epidermis of TGF-β1-null mice displays marked hyperproliferation (Glick *et al*, 1993), whereas mice with targeted overexpression of active TGF-β1 exhibit severely disrupted skin development, leading to neonatal death (Sellheyer *et al*, 1993).

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TGF-β1 is predominantly secreted as a large latent protein complex, consisting of the mature (active) TGF-β1 homodimer, the latency-associated peptide (LAP), and the high molecular weight latent TGF-β binding protein (LTBP) (Wakefield *et al*, 1988; Miyazono *et al*, 1991). Extracellularly, latent TGF-β1 is proteolytically cleaved with the release of active TGF-β1. On the target cell, active TGF-β1 binds to a hetero-tetrameric complex of two transmembrane signaling receptors: TGF-β type I (TβRI) and TGF-β type II (TβRII) receptors (Ebner *et al*, 1993). TβRII phosphorylates TβRI upon binding of TGF-β1 (Wrana *et al*, 1994) and TβRI subsequently phosphorylates either of two intracellular protein homologs, Smad2 or Smad3 (Nakao *et al*, 1997b). Phosphorylated Smad2 and Smad3 associate in the cytoplasm with Smad4 and the resulting complexes translocate to the nucleus, where they modulate transcription in collaboration with other coactivators and corepressors (Massague, 2000). This signaling pathway, however, can be blocked by binding of Smad7 to the intracellular domain of the TβRI, which inhibits the phosphorylation of Smad2 and Smad3 (Nakao *et al*, 1997a).

We have previously reported that ERY OL lesions show epithelial atrophy and hyperproliferation of basal keratinocytes compared with reticular OL and normal oral mucosa (NOM) epithelium (Karatsaidis *et al*, 2003). From studies on transgenic mice with null expression of Smad3, or active TGF-β1 constitutively targeted to the keratin 10 promoter, or overexpression of Smad7 (Cui *et al*, 1995; Ashcroft *et al*, 1999; He *et al*, 2002), it is known that interference with the TGF-β/Smad pathway can cause such changes. In this study, we show that this pathway is inhibited in ERY OL because, concomitantly with an increased activation of TGF-β1, there is an increased Smad7 expression and membrane translocation, and significantly reduced nuclear accumulation of Smad2, Smad3, and Smad4 in the epithelium of ERY OL. Parallel to the findings in transgenic mice, this thus may be the cause for the epithelial hyperproliferation in ERY OL.

MATERIALS AND METHODS

Specimens Biopsies were taken, after informed consent, from volunteers with ERY OL ($n=12$) or with NOM ($n=11$). No distinction was made between OLP and oral lichenoid reactions. Clinical diagnosis was made by an experienced clinician (TA), and pathologic diagnosis was confirmed on hematoxylin and eosin sections of the biopsies. The clinical and histopathologic criteria used were those described by Kramer *et al* (1978). Buccal mucosal biopsies were taken from sites that typified the clinical diagnosis and character of the lesion. The volunteers were tested for oral *Candida* infection using Dentocult dip slides (Orion Diagnostica, Espoo, Finland) and the biopsies were examined by PAS staining. None of the patients included in this study showed *Candida* infection at the time of biopsy. Biopsies were snap-frozen on dry ice (−70°C), oriented, embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and stored at −80°C. Biopsies for *in situ* hybridization were fixed in 4% buffer formaldehyde. Five micron thick sections were cut at −20°C in a cryostat and slides were stored at −20°C until used. The study was carried out according to the Helsinki Declaration's principles for biomedical research and approved by the Ethical Committee of Health, Oslo, Norway.

Immunohistology Antibodies against the following proteins were used in this study: the mature and active TGF-β1 (mouse IgG, 1.25 μg per ml, BioSource International, Camarillo, CA), LAP (TGF-β1) (goat IgG, 1.3 μg per ml, R&D, Oxfordshire, UK), LTBP-1 (rabbit IgG, 0.3 μg per ml, a kind gift from Dr C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden), keratinocyte transglutaminase (mouse IgG, 0.45 μg per ml, Biomedical Technologies, Stoughton, MA). The following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used: TGF-βRI (rabbit IgG, 0.33 μg per ml), TGF-βRII (rabbit IgG, 0.25 μg per ml), phosphorylated Smad2/3 (rabbit IgG, 0.25 μg per ml), Smad4 (mouse IgG, 2 μg per ml), Smad7 (goat IgG, 4 μg per ml), plasminogen activator inhibitor 1 (PAI-1) (mouse IgG, 1 μg per ml).

Single stainings were performed after fixation of cryosections in 4% paraformaldehyde for 20 min, followed by 2 × 5 min washes in phosphate-buffered saline (PBS) and preincubation with 0.3% H_2O_2 for 30 min. Sections were rewashed and incubated in 5% of appropriate normal serum for 30 min (the species of the normal serum used was equivalent to the species of the secondary antibody used; see below). Primary antibodies were diluted in PBS with 1% bovine serum albumin, applied on the sections, and incubated overnight at 4°C. Subsequently, the sections were washed in PBS and incubated 30 min with the appropriate secondary biotinylated antibody preparation (horse antimouse IgG, goat antirabbit IgG, or rabbit antigoat IgG, all diluted 1:200 and all from Vector Laboratories, Burlingame, CA). After washing in PBS, sections were incubated with avidin–biotin complex conjugated with horseradish peroxidase (Vector Laboratories) for 30 min, washed, and finally developed with 3,3′-diaminobenzidine as substrate (Sigma-Aldrich, St Louis, MO).

In double stainings, the sections were fixed as above, washed in PBS, and incubated in 10% normal goat serum and 10% normal horse serum for 30 min. Primary antibodies for keratinocyte transglutaminase and LTBP-1 were diluted and incubated for 1 h. Sections were washed again in PBS followed by incubation of a secondary goat antirabbit biotinylated antibody preparation (1:200, Vector Laboratories) for 30 min. After washing, the sections were incubated in Cy3-labeled streptavidin (1:1000, Amersham Pharmacia Biotech, Uppsala, Sweden) for 30 min. Sections were again washed, followed by sequential incubation with avidin (10 μg per ml) and biotin (1 mg per ml), both from Sigma-Aldrich, for 10 min each, and an incubation of a secondary horse antimouse biotinylated antibody preparation (1:200, Vector Laboratories) for 30 min. This was followed by incubation with Cy2-labeled streptavidin (1:1000, Amersham Pharmacia) for 30 min. Sections were finally washed and counterstained with DAPI (Molecular Probes, Eugene, OR). Isotype-matched control antibodies showed no staining with the immunohistologic detection techniques used.

***In situ* hybridization** After fixation in 4% buffered formaldehyde solution for 24 h, biopsies were processed through graded alcohols, oriented, and embedded in paraffin. Four micron thick paraffin sections were cut and mounted on polylysine-coated glass slides. Prior to staining, sections were dewaxed and rehydrated according to standard procedures. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. The sections were then rehydrated with distilled water and equilibrated in TE-buffer (10 mM Tris–HCl, 1 mM ethylenediamine tetraacetic acid, pH 7.6). Permeabilization was performed with proteinase K (Sigma-Aldrich, 20 μg per ml in TE-buffer, 30 min, 37°C). Proteolytic activity was stopped by incubation with glycine in PBS (2 mg per ml). Sections were postfixed in 4% paraformaldehyde in PBS for 15 min. Acetylation was done with freshly prepared triethanolamine buffer (100 mM, pH 8) with 0.5% acetic anhydride (2 × 5 min). Equilibration and prehybridization were carried out with 5 × sodium citrate/chloride buffer (SSC) (10 min, room temperature) and hybridization buffer (Dako; 2 h, 52 °C), (DAKO, Glostrup, Denmark) respectively. For hybridization, sections were incubated for 18 h at 52 °C with biotinylated antisense or sense probe (GreenStar $Biotin_{10}$ oligonucleotide probe; antisense, 48 bp hybridizing to nucleotides 440–487 in the coding sequence of human Smad7; GeneDetect, Auckland, New Zealand) diluted in hybridization buffer (1 μg per ml). Stringency washes were 2 × SSC (1 h, 52°C), 2 × SSC diluted with 50% formamide (20 min, 52°C), 0.1 × SSC (20 min, 52°C). Blocking was done with Tris-buffered saline (TBS; 0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl) with 1% casein (TBS-C). Further incubations and washings were then peroxidase-conjugated rabbit antibiotin antibodies (Dako P5106, 1:100 in TBS-C, 30 min), washing with TBS with 0.05% Tween 20 (TBS-T), biotinyl-tyramide (Dako GenPoint kit K0620; 8 min), washing with PBS-T, alkaline phosphatase-conjugated rabbit antibiotin antibodies (Dako D5107; 1:75 in TBS-C, 30 min). The sections were finally developed with BCIP/NBT substrate (20 min) and counterstained with Nuclear Fast Red. All aqueous solutions for *in situ* hybridizations were prepared with DEPC-distilled water.

Cell countings Images of sections were captured with a digital camera using objectives with 20×, 40×, 60×, or 100× magnification and monitored on a 17-inch computer screen by means of an imaging software program (Soft Imaging System, Münster, Germany). Cell countings were made using objectives with 20× magnification. For ERY OL specimens, the field chosen for counting was located over the inflammatory cell infiltrate. Total numbers of keratinocytes and numbers of immunohistochemically stained nuclei were counted in the entire part of the epithelium visible on the computer screen.

Statistics *t* tests were used to compare differences between groups. Differences were considered to be statistically significant at $p<0.001$.

RESULTS

An overview of the stainings is presented in **Table I**.

In NOM, basal, spinous, and granular cell layers showed perinuclear, nuclear, and cytoplasmic staining for LAP-TGF-β1 that gradually decreased in intensity from the spinous to the outer epithelial surface (**Fig 1*a***). Cytoplasmic staining for LTBP-1 was seen in all epithelial cell layers in NOM, and this was strongest in spinous, granular, and superficial cell areas (**Fig 1*c***). Speckled staining for active TGF-β1 was observed in upper spinous and granular layers (**Fig 1*e*** with enlargement, showing arrow-marked spots). Strong confluent staining for active TGF-β1 was seen in the outermost superficial epithelial cell layer in NOM (**Fig 1*e***). TβRI and TβRII expression was present as a nuclear, a cell membrane, and a cytoplasmic staining in all NOM epithelial cell layers (**Fig 1*g*, *i***). In NOM epithelium, the staining was most intense in basal cell layers for both TβRI and TβRII. The majority of the cells throughout the epithelium showed nuclear and cytoplasmic staining for both phosphorylated Smad2/3 and Smad4 (**Fig 1*k*, *m***, respectively). NOM epithelium showed areas with patched weak or no cytoplasmic staining for Smad7 (**Fig 1*o*** with enlargement). *In situ* hybridization showed only very weak and scattered nuclear and perinuclear Smad7 mRNA in basal keratinocytes of NOM (**Fig 1*q***; antisense). Control hybridization (sense) did not show any staining (**Fig 1*r***).

In ERY OL, basal and lower spinous layers showed cytoplasmic staining for LAP-TGF-β1, which was especially intense around cell nuclei (**Fig 1*b*** with enlargement). Typically, a band-like area with only scattered or absent LAP-TGF-β1 staining was observed in the uppermost spinous and granular cell layers (**Fig 1*b*** with enlargement). There was strong cytoplasmic staining for LTBP-1 in basal and lower spinous cell layers (**Fig 1*d***). In upper spinous and granular epithelial layers, this staining had a marked pericellular and intercellular location (**Fig 1*d*** with enlargement). Active TGF-β1 consistently showed a clear cell membrane/pericellular staining in the granular and upper spinous epithelial layers, but not in the basal cell layers in ERY OL epithelium (**Fig 1*f*** with enlargement). This band of active TGF-β1 staining was complementary to the cell layers that displayed decreased LAP-TGF-β1 staining (compare **Fig 1*b*, *f*** with respective enlargements). The reduced stores of LAP-TGF-β1 may therefore be a direct result of depletion of latent TGF-β1 in these layers as a consequence of increased activation. Cell membrane, cytoplasmic, and intense perinuclear staining for TβRI and TβRII was seen (**Fig 1*h*, *j***, respectively). This staining was strongly reduced or absent in granular and in upper parts of the spinous epithelial cell layers, often in a band-like pattern, similar to that described above for LAP-TGF-β1. Phosphorylated Smad2/3 (**Fig 1*l*** with enlargement) and Smad4 (**Fig 1*n*** with enlargement) stainings revealed significantly reduced numbers of cells with nuclear and/or cytoplasmic staining among keratinocytes in all epithelial layers in OL compared with epithelial cells in NOM (**Table II**, $p \leq 0.001$, *t* test). The Smad7 staining pattern in ERY OL was profoundly altered compared with NOM (**Fig 1*p*** with enlargement). In contrast to the nearly absent Smad7 expression in NOM, basal and suprabasal cell layers in ERY OL showed strong cytoplasmic Smad7 staining. In spinous and granular layers, the Smad7 staining was particularly localized to the cell membrane. Almost all basal keratinocytes showed strong nuclear and perinuclear expression of Smad7 mRNA in ERY OL epithelium (**Fig 1**; antisense). Control hybridization (sense) did not reveal any staining (**Fig 1*t***).

LTBP-1 has been demonstrated to be targeted to elements of the extracellular matrix through cross-linking by the enzyme transglutaminase (Nunes *et al*, 1997; Verderio *et al*, 1999). Double staining for LTBP-1 and keratinocyte transglutaminase in NOM epithelium showed that LTBP-1 was localized preferentially in the cytoplasm and keratinocyte transglutaminase in cell membrane/pericellular epithelial areas (**Fig 1*u***). In contrast, double staining for LTBP-1 and keratinocyte transglutaminase in ERY epithelium showed a cell membrane/pericellular codistribution of these molecules in spinous and granular cell layers (**Fig 1 *(v1–v3)***).

Finally, we carried out staining for PAI-1, which is upregulated by TGF-β through Smad (Dennler *et al*, 1998). NOM epithelium showed ample cytoplasmic and pericellular staining in spinous and granular cell layers (**Fig 1*w***). In contrast, there was very little staining for PAI-1 in ERY OL epithelium, and this was restricted to only scattered epithelial cells (**Fig 1*x***).

In sum, TGF-β1 was strongly activated in parts of the ERY OL epithelium, but the signal transduction through Smad appeared to be blocked by Smad7. This was accompanied by a concomitant decreased expression of PAI-1 in ERY OL epithelium.

Table I. Summary of specific stainings in NOM and ERY OL

		NOM	OL
LAP-TGF-β	Basal/parabasal layers	+++[a]	+++
	Spinous/granular	+++	0/+
LTBP-1	Basal/parabasal layers	++	+++
	Spinous/granular	+++	+++
Active TGF-β	Basal/parabasal layers	0	0
	Spinous/granular	Spots	+++
TβRI	Basal/parabasal layers	+++	+++
	Spinous/granular	++	0/+
TβRII	Basal/parabasal layers	+++	+++
	Spinous/granular	++	0/+
pSmad2/3	Basal/parabasal layers	+++	0/+
	Spinous/granular	+++	0/+
Smad4	Basal/parabasal layers	+++	0/+
	Spinous/granular	+++	0/+
Smad7	Basal/parabasal layers	±	+++
	Spinous/granular	±	Membrane +++
Smad7 mRNA	Basal/parabasal layers	±	+++
	Spinous/granular	0	0
PAI-1	Basal/parabasal layers	++	±
	Spinous/granular	+++	±

[a]0, no staining; ±, very weak staining; +, weak staining; ++, moderate staining; +++, strong staining.

Table II. Percentages of cells with nuclei stained for Smad2/3 and Smad4 in epithelium of NOM and ERY OL (mean ± standard deviation)

	NOM ($n=11$)	OL ($n=12$)
pSmad2/3	60 ± 18	19 ± 11*
Smad4	56 ± 17	20 ± 12*

*Statistical difference between NOM and OL groups ($p < 0.001$, *t* test).

DISCUSSION

The integrity of normal stratified epithelium is maintained by a balance between proliferation of basal epithelial cells and continuous shedding of terminally differentiated epithelial cells (Jetten and Harvat, 1997; Gandarillas, 2000). Epithelial and stromal cells produce an array of cytokines and growth factors that bind to corresponding receptors on epithelial cells, which upon activation can have a wide variety of antagonistic and synergistic effects on keratinocyte proliferation and differentiation (Dotto, 1999; Freedberg *et al*, 2001). In this *in situ* study, we examined the activation status of TGF-β1 and its intracellular signaling pathway mediated through Smad molecules.

In NOM, we disclosed evidence for active nuclear Smad signaling in the epithelium: numerous Smad4 and phosphorylated Smad2/3 positive nuclei were present, with only sparse signs of

Smad7 expression. This accords with observations in normal skin epidermis, where Smad2, Smad3, and Smad4 nuclear staining also was seen in the epidermal basal, spinous, and granular keratinocytes (He *et al*, 2001). The Smad activation indicates that this, by inference, has a physiologic regulatory function of keratinocyte proliferation and differentiation. Several factors can trigger such Smad signaling. (1) Active TGF-β1 could have been a likely activator, but this was only seen at low levels in the uppermost spinous and granular epithelial cell layers and not among basal and suprabasal keratinocytes where it should have been to exert its growth inhibitory function. This accords with observations from normal epidermis where it is also absent (Kane *et al*, 1991). (2) Growth factors that influence epithelial proliferation and differentiation by signaling through MAPK (e.g., epidermal growth factor and hepatocyte growth factor) can mediate activation and nuclear translocation of Smad2 (de Caestecker *et al*, 1998). This type of cross-talk between Smad and MAPK pathways may also exist in NOM epithelium. (3) Signaling could be induced by TGF-β2 and/or TGF-β3 (Paterson *et al*, 2001). This is not likely because TGF-β2 and TGF-β3 are only expressed in the upper spinous and the granular layers in NOM epithelium (own observations, data not shown) where any action on growth regulation of basal cells is unlikely. (4) Signaling could occur through activin, as activin can regulate keratinocyte differentiation (Beer *et al*, 2000) and mediate downstream Smad2/3-induced transcription (Lebrun *et al*, 1999).

In OL, the normal epithelial structure is severely disrupted, with changes such as hyperkeratosis, atrophy, and degeneration of basal cells (Andreasen, 1968; Kramer *et al*, 1978). In addition, the epithelium shows an abnormally high proliferation rate in the basal cell layer in the ERY form of the disease (Karatsaidis *et al*, 2003). Such changes could be caused by inhibition of the growth regulatory activity of TGF-β1. In this study we therefore examined TGF-β1 signaling in ERY OL at the level of production and activation of the ligand, of TβRI and TβRII expression, and of Smad signal transduction, and we conclude the following. (1) Production and activation of TGF-β1 are not deficient in ERY OL because there was ample staining for latent TGF-β1 (as shown by the presence of LAP-TGF-β1) and a strong staining for active TGF-β1 in the upper parts of the spinous layer and in the granular cell layer. The activation markedly exceeded the levels seen in NOM, but TGF-β1 activation was confined to the more superficial differentiating cell layers and was not seen among basal cells where it could have had an antiproliferative effect. (2) TGF-β signaling is probably not obstructed by deficient expression of TβRI and TβRII because the receptors were normally expressed in basal and spinous layers of the ERY OL epithelium. Incidentally, the upper spinous and granular layers showed a reduced TβRI/TβRII expression. This could be a desensitizing/downregulating response to the increased TGF-β1 activity—a mechanism that has been documented *in vitro* (Prime *et al*, 1994; Zwaagstra *et al*, 1999). It has also recently been shown that Smad7 can be involved in degradation of TGF-β receptors at the protein level (Kavsak *et al*, 2000). As Smad7 was located at the level of the cell membrane in the uppermost spinous and granular layers in ERY OL, this also could contribute to the reduced expression of TβRI and TβRII at this location. (3) There appears to be blockage of the TGF-β1 signal through Smad2 and Smad3 in ERY OL, because there were significantly decreased numbers of cells with nuclear accumulation of the signaling molecules Smad2, Smad3, and Smad4 in all epithelial cell layers, concomitant with a marked cell membrane localization of the inhibitory signaling molecule Smad7. Moreover, Smad7 transcription was shown to be significantly upregulated in basal keratinocytes of ERY OL compared with NOM. Blockage of Smad2 and Smad3 phosphorylation by Smad7 may thus be the cause for the increased proliferation rate in ERY OL epithelium. This notion is strongly supported by studies in transgenic mice that show that both Smad3-null mice and mice with overexpression of Smad7 display increased keratinocyte proliferation (Ashcroft *et al*, 1999; He *et al*, 2002). Moreover, transgenic mice with constitutive active TGF-β1 coupled to the keratin 10 promoter (which targets TGF-β1 to suprabasal epithelial cell layers) also show epidermal hyperproliferation, suprabasal TGF-β1 activation, and epithelial atrophy (Cui *et al*, 1995). The findings in the latter model strongly resemble our observations in ERY OL epithelium. It is likely that a signaling blockage by Smad7 was in part responsible for the (then unexpected) increased epithelial proliferation seen in the keratin-10-TGF-β1 transgenic mice.

The site (i.e., the epithelial layers) where TGF-β1 exerts its activity is known to be crucial for the morphology of the epithelium. TGF-β1 activation within the basal cell layer inflicts major damage to the epithelium: e.g., transgenic mice with constitutive expression of active TGF-β1 targeted to basal keratinocytes do not survive because of a total inhibition of basal cell proliferation (Sellheyer *et al*, 1993). Other transgenic strains, where latent TGF-β1 expression was coupled to keratin 14 promoter (which is conditionally activated in basal keratinocytes), showed marked defects in re-epithelialization during wound healing (Yang *et al*, 2001). This may be the reason why TGF-β1 during normal wound healing is activated in a confined compartment of the more superficial epithelial cell layers, at a distance from the proliferative basal cells (Kane *et al*, 1991). The observed restricted activation of TGF-β1 in ERY OL epithelium resembles this pattern. Our stainings for LTBP-1 and keratinocyte transglutaminase expression might give a clue to why this activation shows this confinement. In connective tissue, LTBP-1 has a function in targeting latent TGF-β to the extracellular matrix and transglutaminase contributes to this process because it cross-links LTBP-1 to extracellular matrix elements (Nunes *et al*, 1997). Transglutaminase is not expressed in basal keratinocytes in oral mucosa (Ta *et al*, 1990;present observations) and if transglutaminase and LTBP-1 have a similar TGF-β targeting function in epithelium as in connective tissue, this might explain the confinement of the TGF-β1 activation. Therefore, as long as the TGF-β1 activity remains compartmentalized as in the present ERY OL biopsies, the lesion could be regarded as a wound that is trying to heal normally but is kept in an unresolved state by the chronic inflammatory infiltrate (see below). If TGF-β1, however, would become very strongly activated and affect the basal cell layers, epithelial proliferation could become so severely inhibited that the lesion then perhaps might turn into its ulcerative form.

The epithelial differentiation pattern and cell death mode are disturbed in ERY OL (Karatsaidis *et al*, 2003), which is the underlying cause for the morpho-pathologic changes typical for the disease. Besides inhibiting epithelial growth, TGF-β1 can affect epithelial differentiation by regulating, for example, keratin and integrin expression and apoptosis (Mansbridge and Hanawalt, 1988; Jiang *et al*, 1995; Zambruno *et al*, 1995; Min *et al*, 1999). Altered epidermal differentiation resembling that in OL can be seen in transgenic mice in which the TGF-β signaling pathway was manipulated; mice with overexpressed epidermal Smad7 show acanthosis and hyperkeratosis (He *et al*, 2002) and keratin-10-TGF-β1 transgenic mice in which the epidermis was treated with 12-O-tetradecanoylphorbol-13-acetate (a hyperplasia inducing agent) acquire pronounced epidermal atrophy (Cui *et al*, 1995). It is therefore possible that the detected inhibition of the TGF-β signaling pathway plays a role in the epithelial atrophy in ERY OL (Karatsaidis *et al*, 2003).

Based on prospective follow-up studies of large groups of OLP patients, it has been suggested that OLP can be a premalignant condition because a small fraction of the initial lesions diagnosed as plaque or ERY OLP over time developed into cancer (Holmstrup *et al*, 1988). Loss of Smad and TGF-β receptor signaling has been observed in cancer cells, and this is associated with decreased growth inhibitory control of TGF-β (Pasche, 2001). Even though the TGF-β/Smad signaling blockade was not complete in ERY OL, alterations in this growth inhibitory pathway might perhaps, in concert with other predisposing factors, play a role in dysplastic transformation of ERY OL.

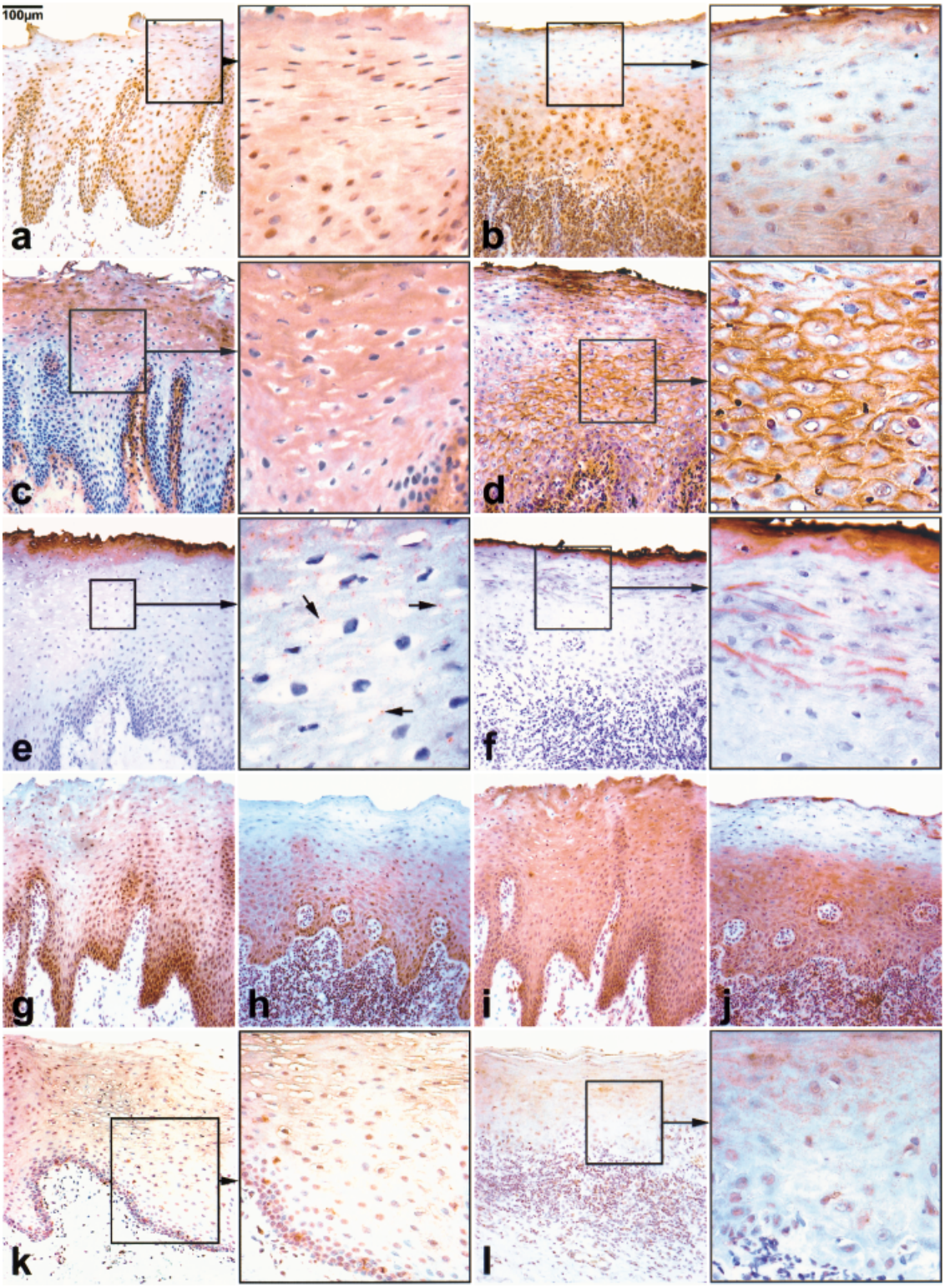

Figure 1. Micrographs of immunohistologically stained tissue sections from biopsies obtained from persons with NOM (*a*, *c*, *e*, *g*, *i*, *k*, *m*, *o*, *u*, *w*) and patients with OL (*b*, *d*, *f*, *h*, *j*, *l*, *n*, *p*, *v*, *x*). After immunohistochemical staining the labeled molecules are seen in *brown* (counterstain with hematoxylin) and for immunofluorescence (*u*, *v*) the specific molecules are displayed in *red* and *green* (overlapping red and green appear *yellow*; *blue* nuclear stain with DAPI). Antibodies used were against (*a*), (*b*) LAP-TGF-β1; (*c*), (*d*) LTBP-1; (*e*), (*f*) active TGF-β1 (*arrows* in (*e*) indicate spotted staining); (*g*), (*h*) TβRI; (*i*), (*j*) TβRII; (*k*), (*l*) phoshorylated Smad2/3; (*m*), (*n*) Smad4; (*o*), (*p*) Smad7; (*q*), (*r*) LTPB-1 in *red*, keratinocyte transglutaminase in *green* (position of the basement membrane indicated with *white broken lines*); (*w*), (*x*) mature PAI-1; *in situ* hybridization for Smad7 (*q*, *r*, *s*, *t*; labeled molecules are in *blue;* counterstain with *red*). Low magnification pictures (lettered *a* to *x*, except for *q*, *r*, *s*, *t*) were obtained with a 20× objective (*scale bar*: 100 µm). High magnification pictures (without lettering) were taken with a 60× objective, except for enlargements (*e*) and (*k*), which were taken with 100× and 40× objectives, respectively. Micrographs of *in situ* hybridization (*q*, *r*, *s*, *t*) were obtained with a 40× objective. Frames of high magnification are connected to their respective low magnification areas with *arrows*. *Scale bar* for fluorescence figures (*u*, *v*) is 10 µm.

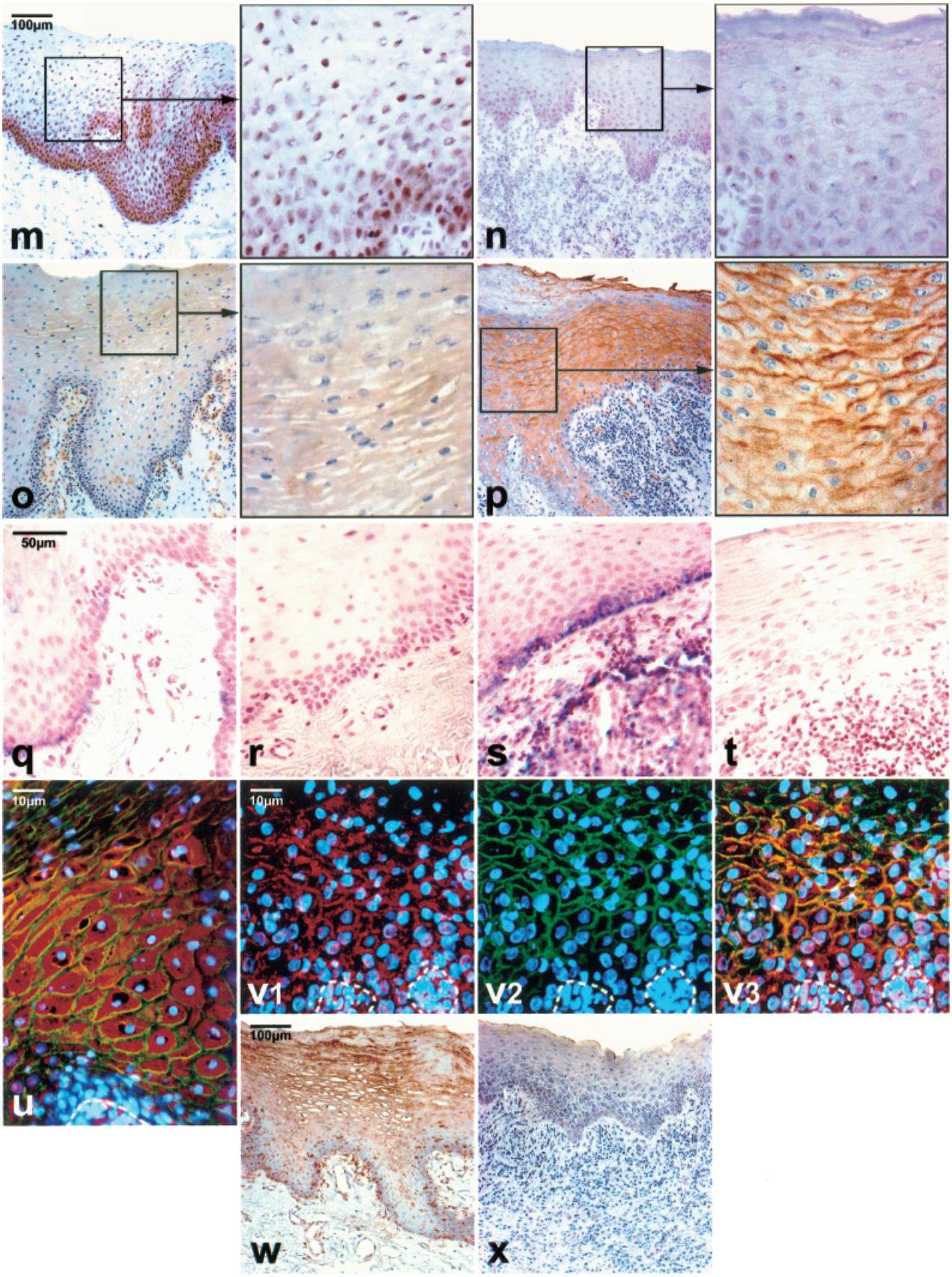

Figure 1. *Continued*

Upregulation of the expression of Smad7 can be caused by pro-inflammatory cytokines like interferon-γ and tumor necrosis factor α (Ulloa *et al*, 1999; Bitzer *et al*, 2000). These are believed to be central regulatory cytokines in OL, and are synthesized and secreted by activated keratinocytes and inflammatory cells in the subepithelial infiltrate (Yamamoto and Osaki, 1995). Typically, Smad7 mRNA was observed in the basal keratinocyte layer in ERY OL. The increased basal cell proliferation in ERY OL epithelium (Karatsaidis *et al*, 2003) can thus be due to inhibition of TGF-β/Smad signaling by Smad7, induced by interferon-γ and tumor necrosis factor α.

TGF-β is a strong upregulator of the transcription of PAI-1 in human keratinocytes (Keski-Oja and Koli, 1992). Elements in the PAI-1 promoter that are binding sites for the Smad3–Smad4 heterodimer have indeed been identified (Dennler *et al*, 1998). PAI-1 expression in ERY OL epithelium was strongly reduced, which indicates a blockage at the functional level of the TGF-β/Smad signaling pathway.

In sum, these results indicate that there is a functional blockage of the TGF-β signaling pathway by increased Smad7 expression and translocation in ERY OL. This may explain the increased hyperproliferation and the disturbed terminal differentiation of keratinocytes observed in this disease.

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REFERENCES

Andreasen JO: Oral lichen planus. 1. A clinical evaluation of 115 cases. *Oral Surg Oral Med Oral Pathol* 25:31–42, 1968

Ashcroft GS, Yang X, Glick AB, *et al*: Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat Cell Biol* 1:260–266, 1999

Axell T, Rundquist L: Oral lichen planus – A demographic study. *Community Dent Oral Epidemiol* 15:52–56, 1987

Beer HD, Gassmann MG, Munz B, Steiling H, Engelhardt F, Bleuel K, Werner S: Expression and function of keratinocyte growth factor and activin in skin morphogenesis and cutaneous wound repair. *J Investig Dermatol Symp Proc* 5:34–39, 2000

Bitzer M, von Gersdorff G, Liang D, Dominguez-Rosales A, Beg AA, Rojkind M, Bottinger EP: A mechanism of suppression of TGF-β/SMAD signaling by NF-κB/Rela. *Genes Dev* 14:187–197, 2000

Bolewska J, Reibel J: T lymphocytes, Langerhans cells and HLA-DR expression on keratinocytes in oral lesions associated with amalgam restorations. *J Oral Pathol Med* 18:525–528, 1989

Bolewska J, Holmstrup P, Moller-Madsen B, Kenrad B, Danscher G: Amalgam associated mercury accumulations in normal oral mucosa, oral mucosal lesions of lichen planus and contact lesions associated with amalgam. *J Oral Pathol Med* 19:39–42, 1990

de Caestecker MP, Parks WT, Frank CJ, Castagnino P, Bottaro DP, Roberts AB, Lechleider RJ: Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases. *Genes Dev* 12:1587–1592, 1998

Cui W, Fowlis DJ, Cousins FM, Duffie E, Bryson S, Balmain A, Akhurst RJ: Concerted action of TGF-β1 and its type II receptor in control of epidermal homeostasis in transgenic mice. *Genes Dev* 9:945–955, 1995

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF: Transforming growth factor-β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci USA* 92:5545–5549, 1995

Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM: Direct binding of Smad3 and Smad4 to critical TGF β-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J* 17:3091–3100, 1998

Dotto GP: Signal transduction pathways controlling the switch between keratinocyte growth and differentiation. *Crit Rev Oral Biol Med* 10:442–457, 1999

Ebner R, Chen RH, Shum L, *et al*: Cloning of a type I TGF-β receptor and its effect on TGF-β binding to the type II receptor. *Science* 260:1344–1348, 1993

Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M: Keratins and the keratinocyte activation cycle. *J Invest Dermatol* 116:633–640, 2001

Gandarillas A: Epidermal differentiation, apoptosis, and senescenc: Common pathways? *Exp Gerontol* 35:53–62, 2000

Glick AB, Kulkarni AB, Tennenbaum T, *et al*: Loss of expression of transforming growth factor-β in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. *Proc Natl Acad Sci USA* 90:6076–6080, 1993

Hannon GJ, Beach D: P15ink4b is a potential effector of TGF-β-induced cell cycle arrest. *Nature* 371:257–261, 1994

He W, Cao T, Smith DA, Myers TE, Wang XJ: Smads mediate signaling of the TGFβ superfamily in normal keratinocytes but are lost during skin chemical carcinogenesis. *Oncogene* 20:471–483, 2001

He W, Li AG, Wang D, *et al*: Overexpression of Smad7 results in severe pathological alterations in multiple epithelial tissues. *EMBO J* 21:2580–2590, 2002

Hedberg N, Ng A, Hunter N: A semi-quantitative assessment of the histopathology of oral lichen planus. *J Oral Pathol* 15:268–272, 1986

Holmstrup P, Thorn JJ, Rindum J, Pindborg JJ: Malignant development of lichen planus-affected oral mucosa. *J Oral Pathol* 17:219–225, 1988

Jetten AM, Harvat BL: Epidermal differentiation and squamous metaplasia: From stem cell to cell death. *J Dermatol* 24:711–725, 1997

Jiang CK, Tomic-Canic M, Lucas DJ, Simon M, Blumenberg M: TGF β promotes the basal phenotype of epidermal keratinocytes: Transcriptional induction of k#5 and k#14 keratin genes. *Growth Factors* 12:87–97, 1995

Kane CJ, Hebda PA, Mansbridge JN, Hanawalt PC: Direct evidence for spatial and temporal regulation of transforming growth factor-β 1 expression during cutaneous wound healing. *J Cell Physiol* 148:157–173, 1991

Karatsaidis A, Schreurs O, Helgeland K, Axell T, Schenck K: Erythematous and reticular forms of oral lichen planus and oral lichenoid reactions differ in pathological features related to disease activity. *J Oral Pathol Med* 32:275–281, 2003

Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL: Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF β receptor for degradation. *Mol Cell* 6:1365–1375, 2000

Keski-Oja J, Koli K: Enhanced production of plasminogen activator activity in human and murine keratinocytes by transforming growth factor-β1. *J Invest Dermatol* 99:193–200, 1992

Kramer IR, Lucas RB, Pindborg JJ, Sobin LH: Definition of leukoplakia and related lesions: An aid to studies on oral precancer. *Oral Surg Oral Med Oral Pathol* 46:518–539, 1978

Lebrun JJ, Takabe K, Chen Y, Vale W: Roles of pathway-specific and inhibitory Smads in activin receptor signaling. *Mol Endocrinol* 13:15–23, 1999

Mansbridge JN, Hanawalt PC: Role of transforming growth factor-β in the maturation of human epidermal keratinocytes. *J Invest Dermatol* 90:336–341, 1988

Massague J: How cells read TGF-β signals. *Nat Rev Mol Cell Biol* 1:169–178, 2000

Min BM, Woo KM, Lee G, Park NH: Terminal differentiation of normal human oral keratinocytes is associated with enhanced cellular TGF-β and phospholipase c-γ1 levels and apoptotic cell death. *Exp Cell Res* 249:377–385, 1999

Miyazono K, Olofsson A, Colosetti P, Heldin CH: A role of the latent TGF-β1-binding protein in the assembly and secretion of TGF-β1. *EMBO J* 10:1091–1101, 1991

Nakao A, Afrakhte M, Moren A, *et al*: Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. *Nature* 389:631–635, 1997a

Nakao A, Imamura T, Souchelnytskyi S, *et al*: TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO J* 16:5353–5362, 1997b

Nunes I, Gleizes PE, Metz CN, Rifkin DB: Latent transforming growth factor-β binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-β. *J Cell Biol* 136:1151–1163, 1997

Pasche B: Role of transforming growth factor-β in cancer. *J Cell Physiol* 186:153–168, 2001

Paterson IC, Matthews JB, Huntley S, Robinson CM, Fahey M, Parkinson EK, Prime SS: Decreased expression of TGF-β cell surface receptors during progression of human oral squamous cell carcinoma. *J Pathol* 193:458–467, 2001

Pietenpol JA, Holt JT, Stein RW, Moses HL: Transforming growth factor β1 suppression of c-myc gene transcription: Role in inhibition of keratinocyte proliferation. *Proc Natl Acad Sci USA* 87:3758–3762, 1990

Prime SS, Matthews JB, Patel V, *et al*: TGF-β receptor regulation mediates the response to exogenous ligand but is independent of the degree of cellular differentiation in human oral keratinocytes. *Int J Cancer* 56:406–412, 1994

Roberts AB: Molecular and cell biology of TGF-β. *Miner Electrolyte Metab* 24:111–119, 1998

Salonen L, Axell T, Hellden L: Occurrence of oral mucosal lesions, the influence of tobacco habits and an estimate of treatment time in an adult Swedish population. *J Oral Pathol Med* 19:170–176, 1990

Sellheyer K, Bickenbach JR, Rothnagel JA, *et al*: Inhibition of skin development by overexpression of transforming growth factor β1 in the epidermis of transgenic mice. *Proc Natl Acad Sci USA* 90:5237–5241, 1993

Ta BM, Gallagher GT, Chakravarty R, Rice RH: Keratinocyte transglutaminase in human skin and oral mucosa: Cytoplasmic localization and uncoupling of differentiation markers. *J Cell Sci* 95:631–638, 1990

Ten Dijke P, Goumans MJ, Itoh F, Itoh S: Regulation of cell proliferation by Smad proteins. *J Cell Physiol* 191:1–16, 2002

Ulloa L, Doody J, Massague J: Inhibition of transforming growth factor-β/SMAD signalling by the interferon-γ/STAT pathway. *Nature* 397:710–713, 1999

Verderio E, Gaudry C, Gross S, Smith C, Downes S, Griffin M: Regulation of cell surface tissue transglutaminase: Effects on matrix storage of latent transforming growth factor-β binding protein-1. *J Histochem Cytochem* 47:1417–1432, 1999

Wakefield LM, Smith DM, Flanders KC, Sporn MB: Latent transforming growth factor-β from human platelets. *J Biol Chem* 263:7646–7654, 1988

Weinberg RA: The retinoblastoma protein and cell cycle control. *Cell* 81:323–330, 1995

Wrana JL, Attisano L, Wieser R, Ventura F, Massague J: Mechanism of activation of the TGF-β receptor. *Nature* 370:341–347, 1994

Yamamoto T, Osaki T: Characteristic cytokines generated by keratinocytes and mononuclear infiltrates in oral lichen planus. *J Invest Dermatol* 104:784–788, 1995

Yang L, Chan T, Demare J, Iwashina T, Ghahary A, Scott PG, Tredget EE: Healing of burn wounds in transgenic mice overexpressing transforming growth factor-β1 in the epidermis. *Am J Pathol* 159:2147–2157, 2001

Zambruno G, Marchisio PC, Marconi A, Vaschieri C, Melchiori A, De Giannetti A, Luca M: Transforming growth factor-β1 modulates β1 and β5 integrin receptors and induces the *de novo* expression of the αvβ6 heterodimer in normal human keratinocytes: Implications for wound healing. *J Cell Biol* 129:853–865, 1995

Zwaagstra JC, Kassam Z, O'Connor-McCourt MD: Down-regulation of transforming growth factor-β receptors: Cooperativity between the types I, II, and III receptors and modulation at the cell surface. *Exp Cell Res* 252:352–362, 1999